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TOP SECRET

~~ISOLATED, POLYPEPTIDES WHICH BIND TO HLA-A29~~
~~MOLECULES, NUCLEIC ACID, THE MOLECULES~~
~~ENCODING THESE, AND USES THEREOF~~

Sub A2 / Isolated Nucleic Acid molecules which
Encode GAGE Genes and Uses Thereof

RELATED APPLICATION

5 This application is a continuation-in-part of Serial No. 08/531,662 filed September 21, 1995, ^{now U.S. Patent No. 5,888,625} which is a continuation-in-part of copending application Serial No. 08/370,648, filed January 10, 1995, ^{now U.S. Patent No. 5,648,226} which is a continuation in part of copending patent application Serial No. 08/250,162 filed on May 27, 1994, ^{now U.S. Patent No. 5,610,013} which is a continuation-in-part of Serial No. 08/096,039 filed July 22, 1993. All of these applications are incorporated by reference. ^{now abandoned}

FIELD OF THE INVENTION

10 20 This invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor. More particularly, the invention concerns genes, whose tumor rejection antigen precursor is processed, inter alia, into at least one tumor rejection antigen that is presented by HLA-Cw6 molecules. The genes in question do not appear to be related to other known tumor rejection antigen precursor coding sequences. The invention also relates to peptides presented by the HLA-Cw6 molecules, and uses thereof. Also a part of the inventions are peptides presented by HLA-A29 molecules, and uses thereof.

BACKGROUND AND PRIOR ART

20 The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T lymphocyte, or "T cell" response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLAs"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced

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Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and HLA/peptide complexes is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257: 880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992). Also see Engelhard, Ann. Rev. Immunol. 12: 181-207 (1994).

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs cytolytic T lymphocytes, or "CTLs" hereafter. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes. Also, see U.S. Patent Application Serial Number 807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774.

In U.S. Patent Application Serial Number 938,334, now U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, it is explained that the MAGE-1 gene codes for a tumor rejection antigen precursor which is processed to nonapeptides which are presented by the

HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind to one HLA molecule, but not to others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed January 22, 1993, ^{now US Patent No 5,558,995} and incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-C clone 10 molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

U.S. Patent Application Serial Number 994,928, filed December 22, 1992, ^{now abandoned} and incorporated by reference herein teaches that tyrosinase, a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield peptides presented by HLA-A2 molecules.

In U.S. Patent Application Serial Number 08/032,978, filed March 18, 1993, and incorporated by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a non-MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

In U.S. Patent Application Serial Number 08/079,110, filed June 17, 1993 and incorporated by reference herein, an unrelated tumor rejection antigen precursor, the so-called "BAGE" precursor, is described. The BAGE precursor is not related to the MAGE family.

The work which is presented by the papers, patents, and patent applications cited supra deals, in large part, with the MAGE family of genes, and the unrelated BAGE gene. It has not been found, however, that additional tumor rejection antigen precursors are expressed by cells. These tumor rejection antigen precursors are referred to as "GAGE" tumor rejection antigen precursors. They do not show homology to either the MAGE family of genes or the BAGE gene. Thus the present invention relates to genes encoding such TRAPs, the tumor rejection antigen precursors themselves as well as applications of both.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

Xaa Trp Pro Xaa Xaa Xaa Xaa Tyr

(SEQ ID NO: 23)

where Xaa is any amino acid and Xaa_(1,2) means that 1 or 2 amino acids may be N-terminal to the Trp residue. These peptides bind to, and/or are processed to peptides which bind to HLA-A29 molecules.

The invention is elaborated upon further in the disclosure which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth lysis studies using CTL clone 76/6.

Figure 2 shows tumor necrosis factor ("TNF") release assays obtained with various transfectants and controls.

Figure 3 compares lysis induced by cytolytic T lymphocytes of clone CTL 76/6. Peptides of varying length were tested, including SEQ ID NO: 4.

Figure 4 presents an alignment of the cDNAs of the six GAGE genes discussed herein. In the figure, identical regions are surrounded by boxes. Translation initiation sites and stop codons are also indicated. Primers, used in polymerase chain reaction as described in the examples, are indicated by arrows.

Figure 5 sets forth the alignment of deduced amino acid sequences for the members of the GAGE family. Identical regions are shown by boxes, and the antigenic peptide of SEQ ID NO: 4, is shown.

Figure 6 shows the results obtained when each of the GAGE cDNAs was transfected into COS cells, together with HLA-Cw6 cDNA. Twenty-four hours later, samples of CTL 76/6 were added, and TNF release was measured after twenty-four hours.

Figure 7 compares the stimulation of CTL 22/23 by COS-7 cells, transfected with HLA-A29 cDNA, a MAGE, BAGE or GAGE sequence, as shown. Control values are provided by MZ2-MEL.43 and COS cells, as stimulators.

Figure 8 presents results obtained by ^{51}Cr release studies, using various peptides including SEQ ID NO: 22 and various peptides derived therefrom.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

A melanoma cell line, MZ2-MEL was established from melanoma cells taken from patent MZ2, using standard methodologies. This cell line is described, e.g., in PCT Application

PCT/US92/04354, filed May 22, 1992, published November 26, 1992, and incorporated by reference in its entirety. Once the cell line was established, a sample thereof was irradiated, so as to render it non-proliferative. These irradiated cells were then used to isolate cytolytic T cell clones ("CTLs") specific thereto.

5 A sample of peripheral blood mononuclear cells ("PBMCs") was taken from patent MZ2, and contacted to the irradiated melanoma cells. The mixture was observed for lysis of the melanoma cells, which indicated that CTLs specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

10 The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39: 390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in vitro, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μ Ci/ml of Na(51 Cr)O $_4$. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10mM Hepes and 10% FCS, after which 100 ul aliquots containing 10^3 cells, were distributed into 96 well microplates. Samples of PBLs were added in 100 ul of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in an 8% CO $_2$ atmosphere.

Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted.

20 Percentage of 51 Cr release was calculated as follows:

$$\% \text{ } ^{51}\text{Cr release} = \frac{(\text{ER-SR})}{(\text{MR-SR})} \times 100$$

where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology. The CTL clone MZ2-CTL 76/6 was thus isolated. The clone is referred to as "76/6" hereafter.

The same method was used to test target K562 cells, as well as the melanoma cell line. Figure 1 shows that this CTL clone recognizes and lyses the melanoma cell line, i.e., MZ2-MEL but not K562. The clone was then tested against other melanoma cell lines and autologous EBV-transformed B cells in the same manner described supra. Figure 1 shows that autologous B cells, transformed by Epstein Barr Virus ("EBV") were not lysed, and that while MZ2-MEL 3.0 was lysed by CTL clone 76/6, the cell line MZ2-MEL.4F, a variant which does not express antigen F, was not. Hence, the clone appears to be specific for this antigen.

The results presented supra are inconclusive as to which HLA molecule presents the TRA. The lysed cell line, i.e., MZ2-MEL, is known to express HLA-A1, HLA-A29, HLA-B37, HLA-B44, HLA-Cw6, and HLA-C clone 10. In experiments not reported here but which followed the protocol of this example, a subline of MZ2-MEL was tested, which had lost expression of HLA molecules A29, B44, and C clone 10. The subline was lysed, thus indicating that the presenting molecule should be one of A1, B37 or Cw6.

Example 2

Further studies were carried out to determine if 76/6 also produced tumor necrosis factor ("TNF") when contacted with target cells. The method used was that described by Traversari et al.,

Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. Briefly, samples of the CTL line were combined with samples of a target cell of interest in culture medium. After 24 hours, supernatant from the cultures was removed, and then tested on TNF-sensitive WEHI cells. Cell line MZ2-MEL.43, a subclone of the MZ2-MEL cell line discussed supra as well as in
5 the cited references, gave an extremely strong response, and was used in the following experiments.

Example 3

The results from Example 2 indicated that MZ2-MEL.43 presented the target antigen of interest. As such, it was used as a source of total mRNA to prepare a cDNA library.

Total RNA was isolated from the cell line. The mRNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the mRNA was secured, it was transcribed into cDNA, via reverse transcription, using an oligo dT primer containing a NotI site, followed by second strand synthesis. The cDNA was then ligated to a BstXI adaptor, digested with NotI, size fractionated by a Sephacryl S-500 HR column, and then cloned, unidirectionally, into the BstXI and NotI sites of pcDNA I/Amp. The recombinant plasmid was then electroporated into DH5α E. coli bacteria. A total of 1500 pools of 100 recombinant bacteria were seeded in microwells. Each contained about 100 cDNAs, because nearly all bacteria contained an insert.

Each pool was amplified to saturation and plasmid DNA was extracted by alkaline lysis and potassium acetate precipitation, without phenol extraction.

Example 4

Following preparation of the library described in Example 3, the cDNA was transfected into eukaryotic cells. The transfections, described herein, were carried out in duplicate. Samples of COS-

7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 50 μ l/well of DMEM medium containing 10% Nu serum, 400 μ g/ml DEAE-dextran, and 100 μ M chloroquine, plus 100 ng of the plasmids. As was indicated supra, the lysis studies did not establish which HLA molecule presented the antigen. As a result, cDNA for each of the HLA molecules which could present the antigen (A1, B37, Cw6) was used, separately, to cotransfect the cells. Specifically, one of 28 ng of the gene encoding HLA-A1, cloned into pCD-SR α , 50 ng of cDNA for HLA-B37 in pcDNA I/Amp, or 75 ng of cDNA for HLA-Cw6 in pcDNAI-Amp, using the same protocol as were used for transfection with the library.

Transfection was carried out in duplicate wells, but only 500 pools of the HLA-Cw6 transfectants could be tested in single wells. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 μ l of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 μ l of DMEM supplemented with 10% FCS.

Following this change in medium, COS cells were incubated for 24-48 hours at 37°C. Medium was then discarded, and 1000-3000 cells of CTL clone 76/6 were added, in 100 μ l of Iscove's medium containing 10% pooled human serum supplemented with 20-30 U/ml of recombinant IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference.

The 1500 pools transfected with HLA-A1, and the 1500 pools transfected with HLA-B37 stimulated TNF release to a concentration of 15-20 pg/ml, or 2-6 pg/ml, respectively. Most of the

HLA-Cw6 transfectants yielded 3-20 pg/ml, except for one pool, which yielded more than 60 pg/ml.

This pool was selected for further work.

Example 5

5 The bacteria of the selected pool were cloned, and 600 clones were tested. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL clone 76/6. Ninety-four positive clones were found. One of these, referred to as cDNA clone 2D6, was tested further. In a comparative test COS cells were transfected with cDNA clone 2D6 and the HLA-Cw6 cDNA, HLA-Cw6 cDNA alone, or cDNA 2D6 alone. Control cell lines MZ2-MEL F and MZ2-MEL F⁺ were also used. TNF release into CTL supernatant was measured by testing it on WEHI cells, as referred to supra. The number of surviving WEHI cells was measured by optical density after incubation of the cells with MTT. Figure 2 shows that the COS cells transfected with HLA-Cw6 and cDNA-2D6, and the cell line MZ2-MEL F⁺ stimulated TNF release from CTL clone 76/6, indicating that HLA-Cw6 presented the subject TRA.

Example 6

20 The cDNA 2D6 was sequenced following art known techniques. A sequence search revealed that the plasmid insert showed no homology to known genes or proteins. SEQ ID NO: 1 presents cDNA nucleotide information for the identified gene, referred to hereafter as "GAGE". A putative open reading frame is located at bases 51-467 of the molecule. The first two bases of this sequence are from the vector carrying the cDNA sequence, and are thus not part of the cDNA itself.

Example 7

Following sequencing of the cDNA, as per Example 6, experiments were carried out to determine if cells of normal tissues expressed the gene. To determine this, Northern blotting was carried out on tissues and tumor cell lines, as indicated below. The blotting experiments used cDNA for the complete sequence of SEQ ID NO: 1. PCT was then used to confirm the results.

Table 1. Expression of gene GAGE

Normal tissues

| | |
|-----------------------|---|
| PHA activated T cells | - |
| CTL clone 82/30 | - |
| Liver | - |
| Muscle | - |
| Lung | - |
| Brain | - |
| Kidney | - |
| Placenta | - |
| Heart | - |
| Skin | - |
| Testis | + |

Tumor cell lines

| | |
|-----------------------------|------|
| Melanoma | 7/16 |
| Lung carcinoma | 1/6 |
| Sarcoma | 0/1 |
| Thyroid medullary carcinoma | 0/1 |

Tumor samples

| | |
|----------|-----|
| Melanoma | 1/1 |
|----------|-----|

Example 8

Detailed analysis of normal tissues and tumors was carried out by applying polymerase chain reaction ("PCR") and the GAGE gene information described supra.

First, total RNA was taken from the particular sample, using art recognized techniques. This was used to prepare cDNA. The protocol used to make the cDNA involved combining 4 ul of reverse transcriptase buffer 5x, 1 ul of each dNTP, (10 mM), 2 ul of dithiothreitol (100 mM), 2 ul of dT-15 primer (20 um), 0.5 ul of RNasin (40 units/ul), and 1 ul of MoMLV reverse transcriptase (200 units/ul). Next, 6.5 ul of template RNA (1 ug/3.25 ul water, or 2 ug total template RNA) was added. The total volume of the mixture was 20 ul. This was mixed and incubated at 42°C for 60 minutes, after which it was chilled on ice. A total of 80 ul of water was then added, to 100 ul total. This mixture was stored at -20°C until used in PCR.

To carry out PCR, the primers

5'-AGA CGC TAC GTA GAG CCT-3'

(sense)

and

5'-CCA TCA GGA CCA TCT TCA-3'

(antisense)

SEQ ID NOS: 2 and 3, respectively, were used. The reagents included 30.5 ul water, 5 ul of PCR buffer 10x, 1 ul of each dNTP (10 uM), 2.5 ul of each primer (20 uM), and 0.5 ul of polymerizing enzyme Dynazyme (2 units/ul). The total volume was 45 ul. A total of 5 ul of cDNA was added (this corresponded to 100 ng total RNA). The mixture was combined, and layered with one drop of mineral oil. The mixture was transferred to a thermocycler block, preheated to 94°C, and amplification was carried out for 30 cycles, each cycle consisting of the following:

first denaturation: 94°C, 4 min.
denaturation: 94°C, 1 min.
annealing: 55°C, 2 min.
extension: 72°C, 3 min.
5 final extension: 72°C, 15 min.

Following the cycling, 10 ul aliquots were run on a 1.5% agarose gel, stained with ethidium bromide.

cDNA amplified using the primers set forth supra yields a 238 base pair fragment. There is no amplification of contaminating genomic DNA, if present.

The results are presented in Table 2, which follows. They confirm that the only normal tissue which expresses GAGE is testis, whereas a number of tumors, including melanoma, lung, breast, larynx, pharynx, sarcoma, testicular seminoma, bladder and colon express the gene. Thus, any one of these tumors can be assayed for expression of the GAGE gene.

Table 2. RT-PCR Analysis of the Expression
of Gene GAGE

| | | | |
|----------------|------------------------|------------|---------------|
| NORMAL TISSUES | | | |
| 5 | Heart | | - |
| | Brain | | - |
| | Liver | | - |
| | Lung | | - |
| | Kidney | | - |
| 10 | Spleen | - | - |
| | Lymphocytes | | - |
| | Bone marrow | | - |
| | Skin | | - |
| | Naevus | | - |
| 15 | Melanocytes | | - |
| | Fibroblasts | | - |
| | Prostate | | - |
| | Testis | | + |
| | Ovary | | - |
| 20 | Breast | | - |
| | Adrenals | | - |
| | Muscle | | - |
| | Placenta | | - |
| | Umbilical cord | | - |
| TUMORS | | | |
| | | Cell Lines | Tumor Samples |
| | Melanoma | 40/63 | 46/146 (32%) |
| | Lung cancer | | |
| 30 | Epidermoid carcinoma | | 10/41 (24%) |
| | Adenocarcinoma | | 4/18 |
| | Small Cell Lung Cancer | 6/23 | 0/2 |
| | Breast cancer | | 15/146 (10%) |
| | Head and neck tumor | | |
| 35 | Larynx | | 6/15 (40%) |
| | Pharynx | | 3/13 |
| | Sarcoma | 1/4 | 6/18 (33%) |
| | Testicular seminoma | | 6/6 (100%) |
| | Bladder cancer | | 5/37 (14%) |
| 40 | Prostate cancer | | 2/20 |
| | Colon cancer | 5/13 | 0/38 |
| | Renal cancer | 0/6 | 0/45 |
| | Leukemia | 3/6 | 0/19 |

Example 9

The identification of the nucleic acid molecule referred to in the prior examples led to further work directed to determination of tumor rejection antigens presented by HLA-Cw6 molecules, and derived from the GAGE gene.

5 The complete cDNA of GAGE in expression vector pcDNA/Amp was digested with restriction endonucleases NotI and SpHI, and then with exonuclease III following supplier's instruction (Erase-a-base System, Promega). This treatment generated a series of progressive deletions, starting at the 3' end.

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The deletion products were ligated back into pcDNA1/^{Amp}~~AMP~~, and then electroporated into E. coli strain DH5 α 'IQ, using well known techniques. The transformants were selected with ampicillin (50 micrograms/ml).

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Plasmid DNA was extracted from each recombinant clone and was then transfected into COS-7 cells, together with a vector which coded for HLA-Cw6. The protocols used ^{followed}~~follow~~ the protocols described above.

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The transfectants were then tested in the TNF release assay. This permitted separation of positive and negative clones. All the negative clones showed a deletion of the entire GAGE sequence. The smallest positive clone contained the first 170 nucleotides of SEQ ID NO: 1. The analysis of this sequence, supra, notes that the open reading frame starts at nucleotide 51. Thus, this fragment contains a sequence which encodes the first 40 amino acids of the GAGE TRAP.

20 Example 10

Additional experiments were then carried out to define the region encoding the TRA peptide more precisely. Polymerase chain reaction ("PCR") amplification was used to do this.

Two primers were synthesized. The first primer was a 22-mer complementary to a sequence within the plasmid vector pcDNA1/Amp located upstream of a BamHI site. The second primer was a 29-mer containing at the 3' end nucleotides 102-119 of SEQ ID NO: 1, and at the 5' end an extension of 11 nucleotides containing an XbaI restriction site.

Following amplification, the PCR product was digested by BamHI and XbaI, and cloned into the BamHI-XbaI sites of plasmid pcDNA-3. The recombinant colonies were cotransfected into COS-7 cells with cDNA encoding HLA-Cw6, in accordance with Example 4, and a TNF release assay, also as described supra, was carried out, using CTL 76/6.

TNF release was observed, indicating that the "minigene" was processed to a TRA. The minigene, i.e., nucleotides 1-119 of SEQ ID NO: 1, the coding region of which runs from nucleotides 51-119, encoded the first 23 amino acids of the cDNA of SEQ ID NO: 1. This information served as the basis for the next set of experiments.

Example 11

Two peptides were synthesized, based upon the first 23 amino acids of SEQ ID NO: 1. These were:

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 12)

and

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val Glu Pro Pro Glu Met Ile
(SEQ ID NO: 13)

Each peptide was pulsed into COS-7 cells previously transfected with HLA-Cw6 cDNA, and combined with CTL 76/6 to determine if TNF release would be induced. Peptides (20 ug/ml) were added to COS-7 cells which had been transfected with the HLA-Cw6 cDNA twenty-four hours

previously After incubation at 37°C for 90 minutes, medium was discarded, and 3000 CTLs were added in 100 microliters of medium, containing 25 units/ml of IL-2. Eighteen hours later, TNF content of supernatant was tested via determining toxicity on WEHI-164-13 cells. The second peptide (SEQ ID NO: 13) was found to induce more than 30 pg/ml of TNF, while the first peptide (SEQ ID NO: 12) was found to induce less than 10 pg/ml of TNF. The second peptide was used for further experiments.

Example 12

Various peptides based upon SEQ ID NO: 13 were synthesized, and tested, some of which are presented below. To carry out these tests ⁵¹Cr labelled LB33-EBV cells, which are HLA-Cw6 positive, were incubated with one of the following peptides:

Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 4)

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 5)

Thr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 6)

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 7)

Arg Pro Arg Pro Arg Arg Tyr Val Glu
(SEQ ID NO: 8)

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 12)

The peptide concentration varied, as indicated in figure 3, and the ratio of CTL: LB33-EBV ("effector: target ratio"), was 10:1. ⁵¹Cr release was determined after four hours of incubation at

37°C. Levels of lysis for positive ("F⁺", MZ2-MEL.3.1), and negative ("F"; MZ2-MEL.2.2.5) control cells are indicated, in figure 3.

It was found, quite surprisingly that the octamer of SEQ ID NO: 4 was the best peptide, and appeared to be the tumor rejection antigen. This is the first time an octamer has been reported as being involved in presentation by a human MHC molecule. There is some precedent for a murine system, as reported by Engelhard, supra, at 199, for H-2K^b and H-2K^K molecules. The nonamers of SEQ ID NO: 5 and SEQ ID NO: 6 also induced CTL lysis albeit to a lesser extent than the octamer of SEQ ID NO: 4.

In results not reported here, a second CTL was tested (CTL 82/31). This CTL was known to lyse cells presenting MZ2-F. It, too, lysed HLA-Cw6 positive cells following pulsing with the peptide of SEQ ID NO: 4.

Example 13

To find out whether the GAGE DNA set forth supra was unique, a cDNA library made with RNA from MZ2-MEL.43 (the same library that was used for the cloning of GAGE) was hybridized with a probe derived from the GAGE cDNA. The probe was a PCR fragment of 308 base pairs between positions 20 and 328 of SEQ ID NO: 1. Twenty positive cDNAs were obtained. Six of them were entirely sequenced. They were all highly related to the GAGE sequence, but they were slightly different from it. Two of the six clones were identical to each other, but all the others differed from each other. Thus, five new sequences different from but highly related to GAGE were identified. They are called GAGE-2, 3, 4, 5 and 6 (Figure 4) and are presented as SEQ ID NOS: 14-

18, respectively. The fourteen other clones were partially sequenced at the 5' end and their sequence corresponded to one of the six GAGE cDNAs.

The major difference between these cDNAs and GAGE-1 is the absence of a stretch of 143 bases located at position 379 to 521 of the GAGE sequence of SEQ ID NO: 1. The rest of the sequences shows mismatches only at 19 different positions, with the exception of GAGE-3 whose 5' end is totally different from the other GAGE for the first 112 bases. This region of the GAGE-3 cDNA contains a long repeat and a hairpin structure.

The deduced GAGE-1 protein corresponding to a tumor rejection antigen precursor is about 20 amino acids longer than the 5 other proteins, whose last seven residues also differ from the homologous residues of GAGE-1 (Figure 5). The rest of the protein sequences show only 10 mismatches. One of these is in the region corresponding to the antigenic peptide of SEQ ID NO: 4. The sequence of the peptide is modified in GAGE-3, 4 5 and 6 so that position 2 is now W instead of R.

Example 14

To assess whether the change at position 2 affected the antigenicity of the peptide, cDNA of the 6 GAGE cDNAs were individually transfected into COS cells together with the cDNA of HLA-Cw6, and the transfectants were tested for recognition by CTL 76/6 as described, supra. Only GAGE-1 and GAGE-2 transfected cells were recognized, showing that the modified peptide encoded by GAGE-3, 4, 5 and 6 was not antigenic in the context of this experiment. Sequence analysis of the 5' end of the 14 other clones mentioned supra showed that 7 of them contained the sequence encoding the antigenic peptide, and thus probably corresponded to either GAGE-1 or GAGE-2.

Example 15

The PCR primers used supra to test the expression of GAGE in tumor samples do not discriminate between GAGE-1 or 2 and the four other GAGE cDNAs that do not encode antigen MZ2F. A new set of primers was prepared which specifically amplifies GAGE-1 and 2, and not GAGE-3, 4, 5 and 6. These primers are:

VDE44 5'-GAC CAA GAC GCT ACG TAG-3' (SEQ ID NO: 9)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3' (SEQ ID NO: 10)

These primers were used as described, supra, in a RT-PCR reaction using a polymerase enzyme in the following temperature conditions:

| | |
|----------------|---------------|
| 40 min at 94°C | |
| 30 cycles with | 1 min at 94°C |
| | 2 min at 56°C |
| | 3 min at 72°C |
| 15 min at 72°C | |

The results of this analysis are set forth in Table 3.

Table 3

Expression of GAGE genes by tumor samples and tumor cell lines

| 5 | Histological type | Number of GAGE positive tumors | |
|----|-------------------------|--------------------------------|--------------|
| | | All GAGE genes* GAGE-1 and 2** | |
| 10 | <u>Tumor samples</u> | | |
| | Melanomas | | |
| | primary lesions | 5/39 | 5/39 (13%) |
| | metastases | 47/132 | 36/131 (27%) |
| 15 | Sarcomas | 6/20 | 6/20 (30%) |
| | Lung carcinoma NSCLC | 14/65 | 12/64 (19%) |
| | Head and neck squamous | | |
| | cell carcinomas | 13/55 | 10/54 (19%) |
| | Prostatic carcinomas | 2/20 | 2/20 |
| | Mammary carcinomas | 18/162 | 14/162 (9%) |
| | Bladder carcinomas | | |
| | superficial | 1/20 | 1/20 |
| | infiltrating | 5/26 | 3/26 |
| | Testicular seminomas | 6/6 | 5/6 |
| | Colorectal carcinomas | 0/43 | |
| | Leukemias and lymphomas | 0/25 | |
| | Renal carcinomas | 0/46 | |
| | <u>Tumor cell lines</u> | | |
| | Melanomas | 45/74 | 40/74 (54%) |
| | Sarcomas | 1/4 | 1/4 |
| | Lung carcinomas | | |
| | SCLC | 7/24 | 7/24 (29%) |
| | NSCLC | 1/2 | 1/2 |
| | Mesotheliomas | 5/19 | 5/19 (26%) |
| | Head and neck squamous | | |
| | cell carcinomas | 0/2 | |
| | Mammary carcinomas | 1/4 | 0/4 |
| 40 | Bladder carcinomas | 0/3 | |
| | Colon carcinomas | 5/13 | 5/13 |
| | Leukemias | 3/6 | 1/6 |
| | Lymphomas | 0/6 | |
| 45 | Renal carcinomas | 0/6 | |

*Expression of GAGE was tested by RT-PCR on total RNA with primers VDE-18 and VDE-24, detecting all GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

**Expression of GAGE-1 and 2 was tested by RT-PCR on total RNA with primers VDE-44 and VDE-24, which distinguish GAGE-1 and 2 from the four other GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

In further work, new primers were designed which amplified all GAGE genes, to make sure that there was no expression of any of them in normal tissues. These primers are

VDE43 5'-GCG GCC CGA GCA GTT CA-3' (SEQ ID NO: 11)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3 (SEQ ID NO: 10)

5 These were used exactly as for the PCR using the VDE44 and VDE24 primers. The results are shown in Table 4. They confirm that the normal tissues are negative, except for testis.

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Table 4

Expression of GAGE genes
in normal adult and fetal tissues

| 5 | Adult tissues | GAGE expression* |
|----|----------------|---------------------|
| 10 | Adrenal gland | - |
| | Benign naevus | - |
| | Bone marrow | - |
| | Brain | - |
| 15 | Breast | - |
| | Cerebellum | - |
| | Colon | - |
| | Heart | - |
| | Kidney | - |
| | Liver | - |
| | Lung | - |
| | Melanocytes | - |
| | Muscle | - |
| | Ovary | - |
| | Prostate | - |
| | Skin | - |
| | Splenocytes | - |
| | Stomach | - |
| | Testis | + |
| | Thymocytes | - |
| | Urinal bladder | - |
| | Uterus | - |
| | Placenta | - |
| | Umbilical cord | - |
| 35 | Fetal tissues* | |
| 40 | Fibroblasts | - |
| | Brain | - |
| | Liver | - |
| | Spleen | - |
| | Thymus | - |
| 45 | Testis | + |

*Expression of GAGE was tested by RT-PCR amplification on total RNA with primers VDE43 and VDE24 detecting all GAGE genes (Figure 7). Absence of PCR product is indicated by - and presence by +. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

*Fetal tissues derive from fetuses older than 20 weeks.

Example 16

In work not reported here, it had been ascertained that cytolytic T cell clone CTL 22/23 (Van den Eynde, et al., Int. J. Cancer 44: 634-640 (1989), incorporated by reference) did not recognize melanoma cell MZ2-MEL.3.1. This melanoma cell line was reported by Van der Bruggen, et al., Eur. J. Immunol. 24: 2134-2140 (1994), to have lost expression of MHC molecules HLA-A29, HLA-B24, and HLA-Cw*1601. Studies were undertaken to determine if transfection with one of these MHC molecules could render the line sensitive to CTL 22/23. HLA-A29 was the first molecule tested. To do so, poly A⁺ RNA was extracted from HLA-A29⁺ cell line MZ2-MEL.43, using a commercially available extraction kit, and following the manufacturer's instructions. The mRNA was then converted to cDNA, using standard methodologies, size fractionated, and then inserted unidirectionally, into the BstXI and NotI sites of plasmid pcDNA-I/Amp. The plasmids were electroplated into *E. coli* strain DH5 α 5'IQ, and selected with ampicillin (50 μ g/ml). The bacteria were plated onto nitrocellulose filters, and duplicated. The filters were prepared, and hybridized overnight in 6xSSC/0.1% SDS/1x Denhardt's solution at 40°C, using ³²P labelled probe:

5'-ACTCCATGAGGTATTTC-3'

(SEQ ID NO: 19)

The probe is a sequence which surrounds the start codon of most HLA sequences.

The filters were washed twice, at room temperature for 5 minutes each time in 6xSSC, and twice in 6xSSC at 43°C. Positive sequences were then screened with probe:

5'-TTTCACCACATCCGTGT-3'

(SEQ ID NO: 20)

which had been labelled with ³²P. This sequence is specific for HLA-A29, as determined by reference to the Kabat Database of sequences and proteins of immunological interest, incorporated by reference.

1/25
C19

This database is available at the NCBI (USA), or on Web Solte (Internet) WWW.NCBI.NLM.NIH.GOV. The filters were washed twice at room temperature for 5 minutes each time, at 6xSSC, followed by two washes, at 6xSSC (5 minutes per wash), at 42°C.

5 Example 17

Once positive HLA-A29 clones were isolated, these were transfected into COS-7 using the DEAE-dextran chloroquine method out supra. In brief, 1.5×10^4 COS-7 cells were treated with 50ng of plasmid pcDNA-I/Amp containing HLA-A29, and 100 ng of cDNA containing cDNA for one of the GAGE sequences mentioned supra, or one of the prior art MAGE or BAGE sequences in plasmid pcDNA α -I/Amp or pcDSR- α , respectively. The transfectants were then incubated for 24 hours at 37°C.

The transfectants were then tested for their ability to stimulate TNF production by CTLs, using the assay explained at the end of example, 4, supra.

Figure 7, which presents the results of this study, shows that high levels of TNF production were achieved using any of GAGE-3, 4, 5 or 6 and HLA-A29 as transfectants. GAGE-1 and GAGE-2, in contrast, do not stimulate CTL clone 22/23, thus leading to the conclusion that GAGE 3, 4, 5 and 6 are processed to an antigen or antigens presented by HLA-A29 molecules and recognized by CTL 22/23.

20 Example 18

The fact that GAGE-3, 4, 5 and 6 were processed to peptides presented by HLA-A29+ cells, while GAGE-1 and GAGE-2 were not, suggested examination of the deduced amino acid sequences for those common to GAGE 3, 4, 5 and 6 and absent from GAGE-1 and GAGE-2. The sequence:

Arg Ser Thr Tyr Trp Pro Arg Pro Arg Arg Tyr Val Gln

(SEQ ID NO: 21)

was identified. The peptide was synthesized, lyophilized, and then dissolved in 1 volume DMSO, 9 volumes of 10 mM acetic acid in water. This methodology was used for the other peptides synthesized, discussed infra.

The peptide (SEQ ID NO: 21) was tested in a ^{51}Cr release experiment, following the method described supra.

It was found that this peptide did provoke lysis. Successive deletions were prepared, and tested for their ability to provoke lysis, again using the ^{51}Cr lytic assay. This work is depicted in Figure 8. It was found that the shortest peptide to provoke lysis was

Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr

(SEQ ID NO: 22), which is common to all of GAGE-3 through 6. Specifically, amino acids 10-18 of GAGE-3, and amino acids 9-17 of GAGE-4, 5 and 6 correspond to this peptide.

The members of the peptide family shown in Figure 8, and represented, e.g., by SEQ ID NOS: 21 and 22, do not accord with the data presented by Toubert, et al., "HLA-A29 Peptide Binding Motif", Abstract No. 4183, Ninth International Congress of Immunology, July 23-29, 1995, San Francisco, CA, incorporated by reference. According to Toubert, et al., at the least a Phe residue is required at the third position of any peptide which binds to HLA-A29. As is shown herein, such is not the case.

The foregoing examples show the isolation of nucleic acid molecules which code for tumor rejection antigen precursors and tumor rejection antigens. These molecules, however, are not homologous with any of the previously disclosed MAGE and BAGE coding sequences described in the references set forth supra. Hence, one aspect of the invention is an isolated nucleic acid molecule

which comprises the nucleotide sequences set forth in any of SEQ ID NOS: 1-6 as well as fragments thereof, such as nucleotides 1-170, and 51-170 of SEQ ID NO: 1, or any other fragment which is processed to a tumor rejection antigen. The sequences of SEQ ID NOS: 1-6 are neither MAGE nor BAGE coding sequences, as will be seen by comparing those to the sequence of any of these genes as described in the cited references. Also a part of the invention are those nucleic acid molecules which also code for a non-MAGE and non-BAGE tumor rejection antigen precursor but which hybridize to a nucleic acid molecule containing the described nucleotide sequence of SEQ ID NO: 1, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refers to hybridization in 1M NaCl, 1% SDS, and 10% dextran sulfate for 18 hours at 65°C. This is followed by two washes of the filter at room temperature for 5 minutes, in 2xSSC, and one wash for 30 minutes in 2xSSC, 0.1% SDS, at 65°C. There are other conditions, reagents, and so forth which can be used, which result in the same or higher degree of stringency. The skilled artisan will be familiar with such conditions and, thus, they are not given here.

It will also be seen from the examples that the invention embraces the use of the sequences in expression vectors, as well as to transform or transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., CHO or COS cells). The expression vectors require that the pertinent sequence, i.e., those described supra, be operably linked to a promoter. As it has been found that both of human leukocyte antigens HLA-Cw6 and HLA-A29 present tumor rejection antigens derived from these genes, the expression vector may also include a nucleic acid molecule coding for one of HLA-Cw6 or HLA-A29. In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The tumor rejection antigen precursor coding sequence may be used alone, when, e.g., the host cell already

expresses one or both of HLA-Cw6 and HLA-A29. Of course, there is no limit on the particular host cell which can be used. As the vectors which contain the two coding sequences may be used in HLA-A29 or HLA-Cw6 presenting cells if desired, and the gene for tumor rejection antigen precursor can be used in host cells which do not express HLA-A29 or HLA-Cw6.

5 The invention also embraces so called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

To distinguish the nucleic acid molecules and the TRAPs of the invention from the previously described MAGE and BAGE materials, the invention shall be referred to as the GAGE family of genes and TRAPs. Hence, whenever "GAGE" is used herein, it refers to the tumor rejection antigen precursors coded for by the previously described sequences. "GAGE coding molecule" and similar terms are used to describe the nucleic acid molecules themselves.

The invention as described herein has a number of uses, some of which are described herein. First, the invention permits the artisan to diagnose a disorder such as melanoma, characterized by expression of the TRAP, or presentation of the tumor rejection antigen. These methods involve determining expression of the TRAP gene, and/or TRAs derived therefrom, such as a TRA presented by HLA-Cw6 or HLA-A29. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with
20 labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred. An alternate method for determination is a TNF release assay, of the type described supra. To carry out the assay, it is preferred to make sure that testis cells are not present, as these normally express GAGE. This is not essential, however,

as one can routinely differentiate between testis and other cell types. Also, it is practically impossible to have testis cells present in non-testicular sample.

The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence coded for by any of SEQ ID NOs:

2-6. These isolated molecules when presented as the TRA, or as complexes of TRA and HLA, such as HLA-Cw6 or HLA-A29, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule.

Exemplary adjuvants include Freund's complete and incomplete adjuvant, killed B. pertussis organisms, "BCG", or Bacille Calmette-Guerin, $Al(OH)_3$, muramyl dipeptide and its derivatives, which may be emulsified in metabolizable oils, such as squalene, monophosphoryl lipid A (MPL), keyhole limpet hemocyanin (KLH), saponin extracts such as QA-7, QA-19, and QA-21 (also referred to as QS-21), these having been described in U.S. Patent No. 5,057,540 to Kensil, et al., incorporated by reference, MTP-MF59, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP), the cationic amphiphile DOTMA, the neutral phospholipids such as DOPE, and combinations of these. This listing is by no means comprehensive, and the artisan of ordinary skill will be able to augment this listing. All additional adjuvants are encompassed herein.

In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative transfectants, et cetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to provide a CTL response, or be cells which express both molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular. Melanoma is well known as a cancer of pigment producing cells.

As indicate, supra, tumor rejection antigens, such as the one presented in SEQ ID NO: 4, are also part of the invention. Also a part of the invention are polypeptides, such as molecules containing from 8 to 16 amino acids, where the polypeptides contain the amino acid sequence set forth in SEQ ID NO: 4. As the examples indicate, those peptides which are longer than the octamer of SEQ ID NO: 4 are processed into the tumor rejection antigen of SEQ ID NO: 4 by the HLA-Cw6 presenting cancer cells, and presented thereby. The presentation leads to lysis by cytolytic T lymphocytes present in a body fluid sample contacted to the cells presenting the complex. Similarly, the peptides longer than SEQ ID NO: 22, such as SEQ ID NO: 21, are processed to the appropriate TRA, and are presented by cancer cells, such as HLA-A29 positive cells.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

Xaa Xaa Trp Pro Xaa Xaa Xaa Xaa Tyr

(SEQ ID NO: 23)

or

Xaa Xaa Trp Xaa Arg Xaa Xaa Xaa Tyr

(SEQ ID NO: 24)

or

Xaa Xaa Trp Xaa Xaa Xaa Xaa Arg Tyr

(SEQ ID NO: 25)

where Xaa in each case is any amino acid.

5
123
C11

Especially preferred are peptides which, in accordance with the formula of SEQ ID NOS: 23, 24 and 25 also satisfy one or more of the following criteria: the N-terminal amino acid position is Tyr, second position is Tyr, fourth position is Pro, fifth position is Arg, sixth position is Pro, seventh position is Arg, and eighth position is Arg. Of course, the fourth position is already fixed in SEQ ID NO: 23, the fifth is already fixed in SEQ ID NO: 24, and the eighth in SEQ ID NO: 25. When all of these criteria are satisfied and this peptide consists of 9 amino acids, one has SEQ ID NO: 22. Any or all of the foreign specific alternatives may be combined in the peptides of the claimed invention, subject to the motif of SEQ ID NO: 23, 24 or 25 and the size of 9-16 amino acids. Especially preferred are peptides which are 9-14 amino acids long, and which include SEQ ID NO: 23, 24 or 25 subject to the above preferred alternatives.

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Also a part of the invention are so-called "minigenes", which are isolated nucleic acid molecules which encode any of SEQ ID NOS: 21, 22, 23, 24 or 25 all of the especially preferred embodiments of SEQ ID NO: 23, 24 or 25 being included. There are only a limited number of nucleic acid molecules which can encode, e.g., SEQ ID NO: 21 or 22, and they can all be deduced from the known rules of degeneracy for codons without any difficulty. The use of these minigenes in encoding the peptides of the invention, in both standard in vivo and in vitro methodologies is also encompassed by the invention. These peptides bind to, and/or are processed to peptides which bind to HLA-A29 molecules. The fact that these peptides are processed to the tumor rejection antigen in indicated by the examples.

This property may be exploited in the context of other parameters in confirming diagnosis of pathological conditions, such as cancer, melanoma in particular. For example, the investigator may

study antigens shed into blood or urine, observe physiological changes, and then confirm a diagnosis of melanoma using the CTL proliferation methodologies described herein.

Also a part of the invention are complexes of HLA-A29 molecules, and one of the peptides listed supra, preferably in soluble form. Such soluble complexes can be used, e.g., to determine presence of CTLs in a sample, such as a body fluid sample, by adding the soluble complexes to the sample, and then determining reaction with CTLs. Panning for CTLs using soluble complexes of MHC molecule and peptide is taught by, e.g., Bousso et al., Immunol. Lett. 59(2): 85-91 (1997), incorporated by reference. The complexes are preferably immobilized to facilitate the enrichment of the CTLs. Attention is also drawn to Sakita, et al., J. Immunol. Meth. 192: 105-115 (1996), also incorporated by reference, which shows that such complexes can be used to stimulate CTLs in vivo. This is another feature of the invention. In an especially preferred embodiment, the soluble complexes referred to supra are multimeric, most preferably tetrameric. Altman et al., Science 274: 94-96 (1996), incorporated by reference, describe how such structures can be made.

On their own, peptides in accordance with the invention may be used to carry out HLA-typing assays. It is well known that when a skin graft, organ transplant, etc., is necessary one must perform HLA typing so as to minimize the possibility of graft rejection. The peptides of the invention may be used to determine whether or not an individual is HLA-Cw6 or HLA-A29 positive, so that appropriate donors may be selected. This type of assay is simple to carry out. The peptides of the invention are contacted to a sample of interest, and binding to cells in that sample indicates whether or not the individual from which the sample is taken is HLA-Cw6 or HLA-A29 positive. One may label the peptides themselves, conjugate or otherwise bind them to linkers which are labeled, immobilize them to solid phases, and so forth, so as to optimize such an assay. Other standard methodologies will be clear to the skilled artisan, and need not be presented herein.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system, leading to lysis of TRA presenting cells, such as HLA-A29 or HLA-Cw6 cells. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein, are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Riddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex, where the complex contains the pertinent HLA molecule. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing RNA of the pertinent sequences, in this case a GAGE sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting

cells are lysed by the mixed CTL sample, then it can be assumed that a GAGE derived, tumor rejection antigen is being presented, and the subject is an appropriate candidate for the therapeutic approaches set forth supra.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccina virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into HLAL-Cw6 presenting cells which then present the HLA/peptide complex of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

(1) GENERAL INFORMATION:

- (i) APPLICANTS: Van der Bruggen, Pierre; Van den Eynde, Benoit; DeBacker, Olivier; Boon-Falleur, Thierry
- (ii) TITLE OF INVENTION: Isolated, Polypeptides Which Bind to HLA-A29 Molecules, Nucleic Acid, The Molecules Encoding These, and Uses Thereof
- (iii) NUMBER OF SEQUENCES: 25
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 - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 kb storage
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 646 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | | | | | |
|-------------|------------|------------|--------------|------------|-----|
| CTGCCGTCCG | GACTCTTTTT | CCTCTACTGA | GATTCATCTG | TGTGAAATAT | 50 |
| GAGTTGGCGA | GGAAGATCGA | CCTATCGGCC | TAGACCAAGA | CGCTACGTAG | 100 |
| AGCCTCCTGA | AATGATTGGG | CCTATGCGGC | CCGAGCAGTT | CAGTGATGAA | 150 |
| GTGGAACCAG | CAACACCTGA | AGAAGGGGAA | CCAGCAACTC | AACGTCAGGA | 200 |
| TCCTGCAGCT | GCTCAGGAGG | GAGAGGATGA | GGGAGCATCT | GCAGGTCAAG | 250 |
| GGCCGAAGCC | TGAAGCTGAT | AGCCAGGAAC | AGGGTCACCC | ACAGACTGGG | 300 |
| TGTGAGTGTG | AAGATGGTCC | TGATGGGCAG | GAGATGGACC | CGCCAAATCC | 350 |
| AGAGGAGGTG | AAAACGCCTG | AAGAAGAGAT | GAGGTCTCAC | TATGTTGCCC | 400 |
| AGACTGGGAT | TCTCTGGCTT | TTAATGAACA | ATTGCTTCTT | AAATCTTTCC | 450 |
| CCACGGA AAC | CTTGAGTGAC | TGAAATATCA | AATGGCGAGA | GACCGTTTAG | 500 |
| TTCTATCAT | CTGTGGCATG | TGAAGGGCAA | TCACAGTGTT | AAAAGAAGAC | 550 |
| ATGCTGAAAT | GTTGCAGGCT | GCTCCTATGT | TGGA AAAATTC | TTCATTGAAG | 600 |

- (2) INFORMATION FOR SEQ ID NO: 2:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGACGCTACG TAGAGCCT 18

- (2) INFORMATION FOR SEQ ID NO: 3:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCATCAGGAC CATCTTCA 18

- (2) INFORMATION FOR SEQ ID NO: 4:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Tyr Arg Pro Arg Pro Arg Arg Tyr
5

- (2) INFORMATION FOR SEQ ID NO: 5:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr

5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Tyr Arg Pro Arg Pro Arg Arg Tyr Val

5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val

5

10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Arg Pro Arg Pro Arg Arg Tyr Val Glu

5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GACCAAGACG CTACGTAG 18

- (2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCATCAGGAC CATCTTCA 18

- (2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGGCCCGAG CAGTTCA 17

- (2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
5 10 15

- (2) INFORMATION FOR SEQ ID NO: 13:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val Glu Pro Pro Glu Met Ile
 5 10 15

- (2) INFORMATION FOR SEQ ID NO: 14:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 538 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGCCAGGGA GCTGTGAGGC AGTGCTGTGT GGTTTCCTGCC GTCCGGACTC 50
 TTTTTCCTCT ACTGAGATTC ATCTGTGTGA AATATGAGTT GGCGAGGAAG 100
 ATCGACCTAT CGGCCTAGAC CAAGACGCTA CGTAGAGCCT CCTGAAATGA 150
 TTGGGCCTAT GCGGCCCGAG CAGTTCAGTG ATGAAGTGGA ACCAGCAACA 200
 CCTGAAGAAG GGGAACCAGC AACTCAACGT CAGGATCCTG CAGCTGCTCA 250
 GGAGGGAGAG GATGAGGGAG CATCTGCAGG TCAAGGGCCG AAGCCTGAAG 300
 CTCATAGCCA GGAACAGGGT CACCCACAGA CTGGGTGTGA GTGTGAAGAT 350
 GGTCCTGATG GGCAGGAGAT GGACCCGCCA AATCCAGAGG AGGTGAAAAC 400
 GCCTGAAGAA GGTGAAAAGC AATCACAGTG TAAAAGAAG ACACGTTGAA 450
 ATGATGCAGG CTGCTCCTAT GTTGGAATT TGTTCATTAA AATTCTCCCA 500
 ATAAAGCTTT ACAGCCTTCT GCAAAGAAAA AAAAAAAA 538

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 560 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
CTCATATTTTC ACACAGATGA GTTGGCGAGG AAGATCGACC TATTATTGGT    50
CTAGGCCAAT AATAGGTCGA TCTTCCTCGC CAACTCATAT TTCACACAGA    100
TGAATCTCAG TAGAGGAAAA TCGACCTATT ATTGGCCTAG ACCAAGGCGC    150
TATGTACAGC CTCCTGAAGT GATTGGGCCT ATGCGGCCCC AGCAGTTCAG    200
TGATGAAGTG GAACCAGCAA CACCTGAAGA AGGGGAACCA GCAACTCAAC    250
GTCAGGATCC TGCAGCTGCT CAGGAGGGAG AGGATGAGGG AGCATCTGCA    300
GGTCAAGGGC CGAAGCCTGA AGCTGATAGC CAGGAACAGG GTCACCCACA    350
GACTGGGTGT GAGTGTGAAG ATGGTCCTGA TGGGCAGGAG ATGGACCCGC    400
CAAATCCAGA GGAGGTGAAA ACGCCTGAAG AAGGTGAAAA GCAATCACAG    450
TGTTAAAAGA AGGCACGTTG AAATGATGCA GGCTGCTCCT ATGTTGGAAA    500
TTTGTTTATT AAAATTCTCC CAATAAAGCT TTACAGCCTT CTGCAAAGAA    550
AAAAAAAAAA                                         560
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 540 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
CGCCAGGGAG CTGTGAGGCA GTGCTGTGTG GTTCCTGCCG TCCGGACTCT    50
TTTTCTCTTA CTGAGATTCA TCTGTGTGAA ATATGAGTTG GCGAGGAAGA    100
TCGACCTATT ATTGGCCTAG ACCAAGGCGC TATGTACAGC CTCCTGAAAT    150
GATTGGGCCT ATGCGGCCCC AGCAGTTCAG TGATGAAGTG GAACCAGCAA    200
```

| | | | | | |
|------------|------------|------------|------------|------------|-----|
| CACCTGAAGA | AGGGGAACCA | GCAACTCAAC | GTCAGGATCC | TGCAGCTGCT | 250 |
| CAGGAGGGAG | AGGATGAGGG | AGCATCTGCA | GGTCAAGGGC | CGAAGCCTGA | 300 |
| AGCTGATAGC | CAGGAACAGG | GTCACCCACA | GACTGGGTGT | GAGTGTGAAG | 350 |
| ATGGTCCTGA | TGGGCAGGAG | ATGGACCCGC | CAAATCCAGA | GGAGGTGAAA | 400 |
| ACGCCTGAAG | AAGGTGAAAA | GCAATCACAG | TGTAAAAAGA | AGGCACGTTG | 450 |
| AAATGATGCA | GGCTGCTCCT | ATGTTGGAAA | TTTGTTTATT | AAAATTCTCC | 500 |
| CAATAAAGCT | TTACAGCCTT | CTGCAAAAAA | AAAAAAAAAA | | 540 |

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 532 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

| | | | | | |
|------------|------------|------------|------------|------------|-----|
| AGCTGTGAGG | CAGTGCTGTG | TGGTTCCTGC | CGTCCGGACT | CTTTTTCCTC | 50 |
| TACTGAGATT | CATCTGTGTG | AAATATGAGT | TGGCGAGGAA | GATCGACCTA | 100 |
| TTATTGGCCT | AGACCAAGGC | GCTATGTACA | GCCTCCTGAA | GTGATTGGGC | 150 |
| CTATGCGGCC | CGAGCAGTTC | AGTGATGAAG | TGGAACCAGC | AACACCTGAA | 200 |
| GAAGGGGAAC | CAGCAACTCA | ACGTCAGGAT | CCTGCAGCTG | CTCAGGAGGG | 250 |
| AGAGGATGAG | GGAGCATCTG | CAGGTCAAGG | GCCGAAGCCT | GAAGCTGATA | 300 |
| GCCAGGAACA | GGGTCACCCA | CAGACTGGGT | GTGAGTGTGA | AGATGGTCCT | 350 |
| GATGGGCAGG | AGATGGACCC | GCCAAATCCA | GAGGAGGTGA | AAACGCCTGA | 400 |
| AGAAGGTGAA | AAGCAATCAC | AGTGTTAAAA | GAAGGCACGT | TGAAATGATG | 450 |
| CAGGCTGCTC | CTATGTTGGA | AATTTGTTCA | TTAAAATTCT | CCCAATAAAG | 500 |
| CTTTACAGCC | TTCTGCAAAG | AAAAAAAAAA | AA | | 532 |

- (2) INFORMATION FOR SEQ ID NO: 18:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 539 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

| | | | | | |
|------------|------------|------------|------------|------------|-----|
| GCCAGGGAGC | TGTGAGGCAG | TGCTGTGTGG | TTCCTGCCGT | CCGGACTCTT | 50 |
| TTTCCTCTAC | TGAGATTCAT | CTGTGTGAAA | TATGAGTTGG | CGAGGAAGAT | 100 |
| CGACCTATTA | TTGGCCTAGA | CCAAGGCGCT | ATGTACAGCC | TCCTGAAGTG | 150 |
| ATTGGGCCTA | TGCGGCCCGA | GCAGTTCAGT | GATGAAGTGG | AACCAGCAAC | 200 |
| ACCTGAAGAA | GGGGAACCAG | CAACTCAACG | TCAGGATCCT | GCAGCTGCTC | 250 |
| AGGAGGGAGA | GGATGAGGGA | GCATCTGCAG | GTCAAGGGCC | GAAGCCTGAA | 300 |
| GCTGATAGCC | AGGAACAGGG | TCACCCACAG | ACTGGGTGTG | AGTGTGAAGA | 350 |
| TGGTCCTGAT | GGGCAGGAGG | TGGACCCGCC | AAATCCAGAG | GAGGTGAAAA | 400 |
| CGCCTGAAGA | AGGTGAAAAG | CAATCACAGT | GTAAAAGAA | GACACGTTGA | 450 |
| AATGATGCAG | GCTGCTCCTA | TGTTGGAAAT | TTGTTTATTA | AAATTCTCCC | 500 |
| AATAAAGCTT | TACAGCCTTC | TGCAAAAAAA | AAAAAAA | | 539 |

- (2) INFORMATION FOR SEQ ID NO: 19:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACTCCATGAG GTATTTC

17

- (2) INFORMATION FOR SEQ ID NO: 20:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TTTCACCACA TGCGTGT

17

- (2) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Arg Ser Thr Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr Val Gln
5 10

- (2) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr
5

- (2) INFORMATION FOR SEQ ID NO: 23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ix) FEATURE:
(D) OTHER INFORMATION: Each Xaa may be any amino acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Xaa Xaa Trp Pro Xaa Xaa Xaa Xaa Tyr
5

09782745.071601

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: Each Xaa may be any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Xaa Xaa Trp Xaa Arg Xaa Xaa Xaa Tyr

5

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: Each Xaa may be any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Xaa Xaa Trp Xaa Xaa Xaa Xaa Arg Tyr

5

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